Ontogeny of the circadian clock in *Sarcophaga crassipalpis*

Senior Thesis

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4/14/2016

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I ABSTRACT

The timing of circadian rhythms is controlled by the rhythmic expression of clock genes in pacemaker neurons within the brain. Circadian gene expression and behavior can entrain to thermoperiod and photoperiod. However, the availability of these cues, the organization of the brain, and the need for circadian behavior all change throughout insect metamorphosis. The purpose of this study is to examine how the entrainment and expression of the circadian clock change throughout fly development using *Sarcophaga crassipalpis* as a model organism. The expression of the clock genes *period* and *timeless* during various developmental time points was examined using qRT-PCR to determine the ontogeny of the circadian clock. Our findings indicate that rhythmic clock gene expression ceases during early pharate adulthood, only to resume by the time of eclosion. The entrainment of the clock was also tested by exposing flies to different combinations of thermoperiod and photoperiod and testing the effect on the timing of the circadian event of eclosion. qRT-PCR was used to measure *period* expression under conditions that could and could not entrain eclosion. Thermoperiod entrains expression of *period* and controls the time of eclosion, suggesting that *period* may be the upstream determinant of eclosion. These results indicates patterns of clock gene expression are dynamic through the life history of an animal.

II INTRODUCTION

Plants and animals need to adjust their behavior and physiology to anticipate stresses like temperature, desiccation, and predation that correspond to diel cycles (Watari, 2009; Parecis-

Silva, 2016). The timing of these preparatory changes in physiology and behavior is programmed by the circadian clock. The circadian clock is composed of the genes *period, timeless, clock, cycle, cryptochrome 1,* and *cryptochrome 2,* though *cryptochrome 2* is absent in *Drosophila* (Goto, 2013). These genes form a negative transcriptional feedback loop, resulting in *per* and *tim* mRNA levels that oscillate with peak expression shortly after lights off (Yuan et al., 2007; Gentile et al., 2009; Goto & Denlinger, 2002). While PER and TIM can form a heterodimer which translocates to the nucleus, they can also act independently with PER entering the nucleus before TIM (Rutila et al., 1996; Shafer et al., 2002; Ashmore et al., 2003). PER can repress transcription *in vitro* and *in vivo* without TIM, though the two proteins do influence each other's stability (Rothenfluh et al., 2000; Ashmore et al., 2003; Weber and Kay 2003).

Clock genes are expressed mainly in the brain (Kostal et al., 2009), which undergoes extensive development during metamorphosis (Fraenkel & Hsiao, 1967). To entrain thermoperiod peripheral tissues signal the clock neurons within the brain to entrain to temperature, though entrainment to photoperiod requires no input from peripheral tissue (Sehadova et al., 2009). There is a temperature receptor (Hamada et al., 2008) active in the peripheral tissue that may be able to synchronize the molecular clock with the pacemaker neurons.

A major circadian event in the life history of many dipterans is adult eclosion. The gating of eclosion is controlled by an insect's circadian clock, which is sensitive to both thermoperiod and photoperiod (Myers, 2003). However, *Drosophila melanogaster tim* mutants can still display rhythmic eclosion (Wuelbeck, 2005). This suggesting other genes in the clock are still active, but the clock is functioning according to a model outside of the PER-TIM negative feedback loop. Alternative models for the clock may be necessary because the cues to which a fly is exposed

will change throughout its development, altering whether or not rhythmicity is a selected trait. Many flies burrow underground during the wandering larval stage in order to spend metamorphosis beneath the substrate, where photoperiod will be extremely dampened or absent, and thermoperiod will have a reduced amplitude. During metamorphosis *S. crassipalpis* is immobile and lacks overt behavior, meaning there may be no selective pressure for circadian rhythmicity until shortly before eclosion. In *Delia antiqua* the eclosion rhythm is entrained by thermoperiod during the pharate adult stage, while in the bee *Megachilidae rotundata* there is a direct link between the temperature regime during pupal stages and eclosion time (Watari, 2005; Yocum et al., 2015). Alternatively, the eclosion rhythm may be entrained before pupariation altogether. In *Sarcophaga argyrostoma* photoperiod during larval development determines eclosion rhythm, but during pupal and pharate adult development photoperiod was unable to synchronized eclosion (Saunders, 1979). *Lucilia cuprina* is sensitive to cues entraining eclosion only during its larval development, as is *Sarcophaga crassipalpis* (Smith, 1985; Joplin & Moore, 1999). However this is a point of contention, as Miyazaki et al. (2011) found that temperature oscillations can control the time of adult eclosion in *S. crassipalpis* that have completed diapause.

The mechanism of entrainment is only partially understood. The circadian clock entrains to photoperiod via the action of CRY1, which degrades TIM in the presence of blue light (Yuan et al., 2007). Thermoperiod is a powerful Zeitgeber, with only 2^{o}C amplitude of thermoperiod able to robustly synchronize *Drosophila melanogaster* behavior, and only 0.4 ^{o}C amplitude thermoperiod able to synchronize eclosion in *Glossina morsitans* (Wheeler et al., 1993; Zdarek & Denlinger, 1995). The effect of temperature on the circadian clock genes is less well understood. In *Drosophila*, the clock gene *Drosophila Clock* (*clk)* is necessary to entrain other

clock gene expression to thermoperiod, though *per* seems to function independent of *clk* (Yoshii et al., 2007). In *D. melanogaster* thermoperiod is able to entrain the expression of *per-luc* constructs (Glaser & Stanewsky, 2005). This suggests an important role for *per* in thermoperiod sensitivity. The interaction of thermoperiod and photoperiod to determine eclosion rhythm varies from insect to insect. *Gryllus bimaculatus,* after removal of the optic lobes, has been shown to entrain behavior to changes in temperature (Karpova & Tomioka, 2006). *Chymomyza costata* raised at low temperatures has more synchronous eclosion, but the eclosion rhythm is still determined by photoperiod (Lankinen &Riihimaa, 1996). *Delia antiqua* has an eclosion rhythm that corresponds to both temperature and light cycles interacting in a manner consistent with the two oscillator model (Watari &Tanaka, 2010; Pittendrigh & Bruce, 1959). However, in *Glossina morsitans* thermoperiod overrides photoperiod altogether, suggesting a single oscillator model (Zdarek & Denlinger, 1995).

This study will focus on the model organism *S. crassipalpis,* as it has the genes *period, timeless* and *cryptochrome 1* sequenced and displays rhythmic eclosion (Goto & Denlinger, 2002). I hypothesize the activity of the circadian clock changes throughout the life of *S. crassipalpis*, with limited rhythmicity from pupariation until the early pharate adult stage. This will be reflected in the temporal expression pattern of clock genes. This study uses qRT-PCR to record changes in *per* and *tim* expression during different life stages and correlate the changes in *per* expression to the entrainment of eclosion by thermoperiod and photoperiod. I hypothesize that *per* expression will correspond to whichever cue synchronizes eclosion.

III MATERIALS AND METHODS

Insects

A colony of *S. crassipalpis* Macquart originating from adults collected in Worthington, Ohio in 2013 was maintained as described (Denlinger, 1972). Flies were maintained under 16-hr light and 8-hr darkness at 22ºC. For ontogeny experiments flies larviposited on a piece of beef liver were raised under 16-hr light and 8-hr darkness at 22ºC and collected during the wandering larval stage, the early pharate adult stage corresponding to 7 days after pupariation, the late pharate i.e. black bristle stage (1 day prior to eclosion), then transferred to 11-hr light and 13-hr darkness at 18ºC and collected 14 days after eclosion, after larvipositing. For eclosion experiments flies were reared at 16L:8D until pupariation then placed in one of the five conditions detailed in the section below. Flies were either placed in the experimental apparatus detailed in the section below, or kept in their thermoregime and photoregime until the day before eclosion when flies were collected for qRT-PCR experiments.

Eclosion experiments

The five combinations of thermoperiod and photoperiod to which flies were exposed are as follows: 16L:8D in combination with 16 hours at 25ºC and 8 hours at 18ºC, 16L:8D in combination with 16 hours at 18ºC and 8 hours at 25ºC, 16L:8D in combination with constant 22ºC temperature, constant darkness in combination with 16 hours at 25ºC and 8 hours at 18ºC, constant darkness in combination with constant 22ºC temperature (N=50 for all groups). Two days before adult eclosion flies were transferred from these conditions to constant darkness and 20ºC. In these conditions the puparia were placed in an experimental apparatus that positioned them within 0.2 mL test tubes with a small metal bead at the mouth of the test tube. Eclosing

flies bumped the bead into a funnel which directed the beads to a fractional collector set to rotate every 32 minutes. In this way the rhythmicity of eclosion could be measured without disturbing the flies.

QRT-PCR to determine clock entrainment

One day before eclosion flies were removed from their rearing conditions at ZT0 and ZT16, and frozen at -80 ^{o}C (N=4, each a pooled sample of 3 heads). RNA was extracted, cDNA was synthesized and qRT-PCR was run according to Meuti et al. (2015) with several minor differences in procedure. Neither sodium acetate nor Glycoblue were added during RNA extraction. During cDNA synthesis 1 µ of RNA was used per 20 µl reaction. *per, tim,* and *cry1* were measured with results normalized to *cry1*, which is expressed constitutively in the brain (Goto & Denlinger, 2002; Veleri & Wulbeck, 2004). Primers are listed in Table 1.

Table1

Sequence of primers

Oligonucleotides	Sequence (5'>>3')
S. c. tim1 FWD	CTACCTCGGTTGACAGAACTAAC
S. c. tim1 REV	CCAGTAGAGTGTGTCCATTTC
S. c. per4 FWD	GCACGAATTGGTCGTATTAAAG
S. c. per4 REV	GACACCTTCTAGAAACCTCTTG
S. c. cry1.4 FWD	GGGAACATGGCTTACGATATT
S. c. cry1.4 REV	AAATGCCGACGAACTAACC

QRT-PCR experiments to determine clock ontogeny

At the wandering larval stage, the early pharate adult stage, and the late pharate adult stage flies were collected at ZT0 and ZT16, corresponding with the lights of the incubator turning on and off respectively. The posterior half of the larvae and early pharate adults were removed to allow penetration of RNAlater (Thermofischer) into the tissue. Samples were stored for three weeks or less in 1mL of RNAlater at 4^{o}C, then the brains were dissected in 100% ethanol to collect the central brain and optic lobes (N=4 for all groups, each a pooled sample of either 5 brains or 3 heads). The validity of using only brains preserved in this manner to detect gene expression was checked by also using entire heads of larvae and early pharate adults frozen at -80^{o}C, which yielded the same patterns of statistical significance. For flies collected during the late pharate adult and post larviposition adult stages flies were frozen at -80^{o}C until decapitation and RNA extraction. RNA extraction, cDNA synthesis and qRT-PCR was run in the same manner as the clock entrainment experiments.

IV RESULTS

QRT-PCR experiments to determine clock ontogeny

QRT-PCR of *period* and *timeless* was performed on different life stages of flies collected at ZT0 and ZT16, corresponding to the time of lights on and lights off. The relative biological expression of *per* and *tim* at these life stages are displayed in Table 2. Significant difference was found between the expressions of *tim* at lights on and lights off in 3rd instar larvae, adults 1 day prior to eclosion, and post larviposition adults. Significant difference was found between the expressions of *per* at lights on and lights off in adults 1 day prior to eclosion, and post-larviposition adults (students t test $p<0.05$). There was no significant difference in gene

expression of *tim* between lights on and lights off during the early pharate adult stage. There was no significant difference in gene expression of *per* between lights on and lights off during the wandering 3rd instar stage or the early pharate adult stage.

Life Stage	Relative Biological expression					
	per			tim		
	zt 0	zt 11 or 16	ttest	zt 0	zt 11 or 16	ttest
3rd instar larvae	1.06600	1	0.91256	1	8.95341	0.03337
Early Pharate Adults	1	1.13676	0.58610	1.09896	1	0.90666
Pre-eclosion adults	1	1.30261	0.03242	1	3.45933	0.02483
Post Larviposition Adults	1	4.28027	0.00549	1	2.90283	0.00046

Table 2: This table shows the relative biological expression of the genes *per* and *tim* between lights on and lights off at various life stages. It also shows the student t test value between the lights on and lights off replicates.

Eclosion experiments

The synchrony of eclosion was measured in groups of flies raised under the combinations of thermoperiod and photoperiod described in the methods section. The eclosion pattern under different combinations of thermoperiod and photoperiod is shown in Figures 1 and 2. The Fisher exact test was used to detect significance ($p<.05$) between the number of flies that eclosed within 8 hours of lights on or lights off to test for eclosion synchrony. This test showed statistically significant synchronous eclosion for synchronous thermoperiod and photoperiod, thermoperiod alone, and conflicting photoperiod and thermoperiod. There was no significant synchrony in photoperiod alone or flies without thermoperiod or photoperiod.

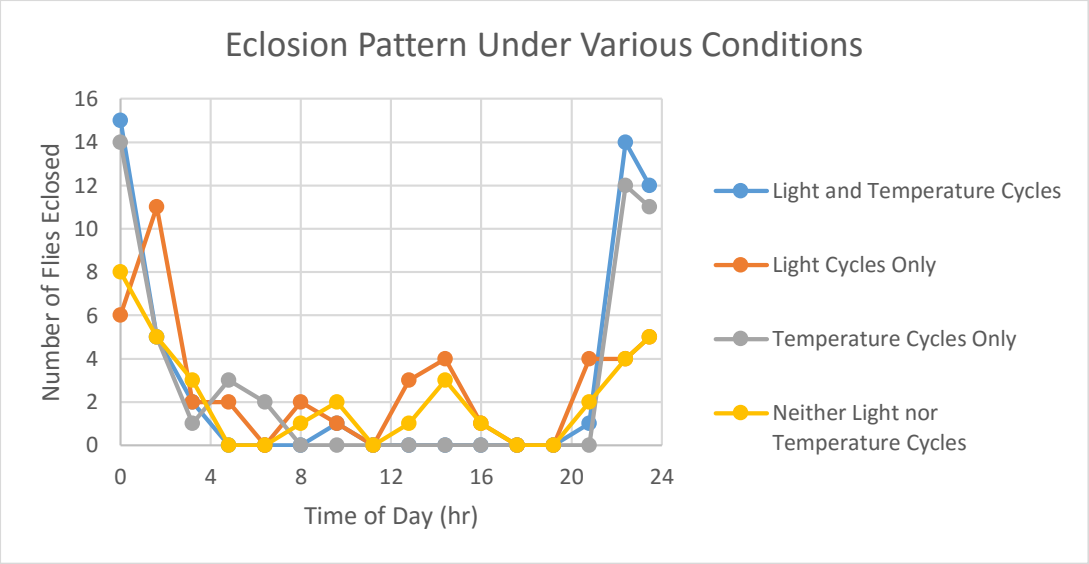

Figure 1: The number of flies eclosed throughout the day under varying temperature and light regimes corresponding to only thermoperiod, only photoperiod, both cues, or neither cue.

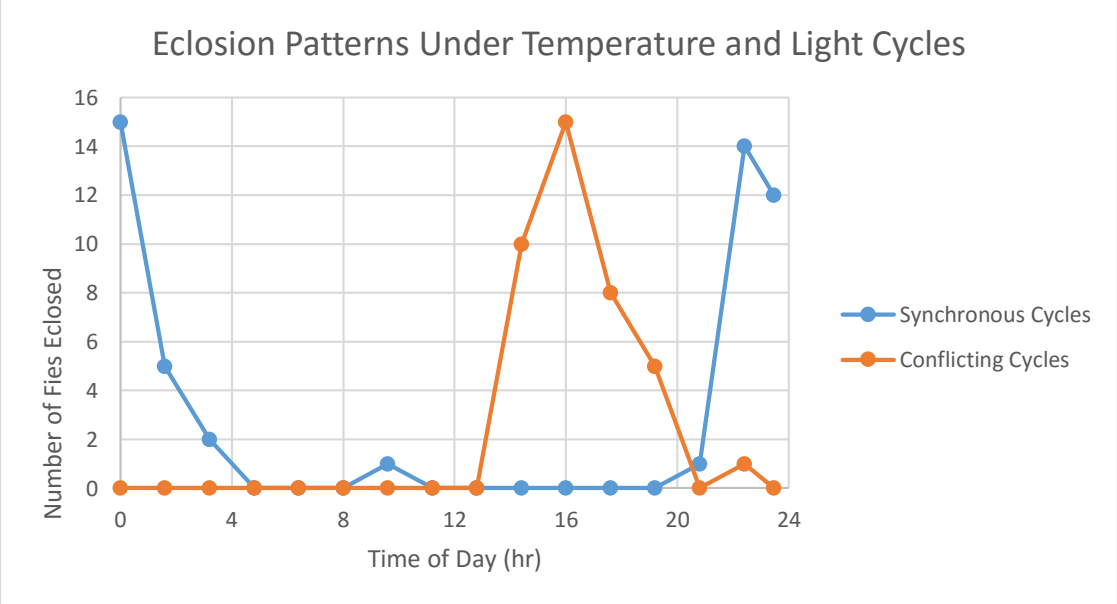

Figure 2: The number of flies eclosing throughout the day under synchronous and conflicting thermoperiod and photoperiod.

QRT-PCR to determine clock entrainment

The effect of thermoperiod and photoperiod was investigated by moving flies to one of five conditions which had a combination of thermoperiod and photoperiod. One day before eclosion the transcription level of *per* was measured using qRT-PCR normalized to *cry1*. Results are indicated in Table 3. Significant difference was present in the transcription level of *per* between lights on and lights off in groups exposed to synchronous thermoperiod and photoperiod, only thermoperiod, and conflicting thermoperiod and photoperiod. The two conditions, photoperiod alone and no thermoperiod combined with no photoperiod, did not significantly entrain *per*.

Conditions during period following pupariation	Relative Biological expression		
	per		
	Zt 0	Zt 16	ttest
Synchronous thermoperiod and photoperiod	1	1.30261	0.03242
Photoperiod alone	1	1.01751	0.95179
Thermoperiod alone	1	2.495812973	0.04187
Conflicting thermoperiod and photoperiod	1.21095	1	0.03149
No thermoperiod or photoperiod	1.09712	1	0.53588

Table 3: This table shows the relative biological expression of the gene *per* between lights on and lights off at under various combinations of thermoperiod and photoperiod. It also shows the student t test value between the lights on and lights off replicates.

V DISCUSSION

Throughout flesh fly development the fly will have different needs for circadian behavior and will be exposed to a variety of heat and light cues. This study sought to measure circadian rhythmicity by using qRT-PCR to measure gene expression at various life stages. There was significant difference in *tim* between lights on and lights off in wandering 3rd instars, but not in *per*. Finding rhythmicity in *tim* expression but not *per* expression was unexpected. Previous research shows periodicity in *per* and *tim* in first instars, while this study found no rhythmicity in pharate adults (Kostal el al., 2009). The loss of rhythmicity is associated with the desynchronization of the pacemaker neurons (Hong & Saunders, 1998). The expression in the wandering third instar may represent a transitional phase as the clock gene expression ceases oscillation, corresponding to the desynchronization of the neurons expressing clock genes. The development of the brain during metamorphosis may lead to rearrangement of neurons and their subsequent desynchronization (Fraenkel & Hsiao, 1967). It's also likely due to a change in which neurons are functional. In the drosophila brain the neurons expressing circadian genes are well mapped out, and they fall into two groups: those active in the first instar, called early pacemaker neurons, and those inactive until the late pupal stages called late pacemaker neurons, compromising 85% of the brains neurons (Kaneko et al., 1997; Kaneko & Hall, 2000). Late pacemaker neurons are present in the third instar larvae expressing CLK and CYC, but are unable at this stage to generate rhythmic *per* and *tim* expression, suggesting that some cue experienced in the late pupal stages allows CLK-CYC and PER-TIM to interact (Liu et al., 2015). *Per* and *tim* expression was uniform between lights on and lights off in pharate adults, but resumed rhythmicity by the day before eclosion, suggesting that this model of pacemaker neuron development may also be applicable to *S. crassipalpis*. Adults also exhibited rhythmic

expression of *per* and *tim*. The rhythmic expression of clock genes in adults just prior to eclosion and after larviposition is not surprising, as circadian genes are linked to rhythmic behavior and eclosion, and adults of *S. crassipalpis* display rhythmic behavior (Smith & Konopka, 1982; Seghal et al., 1994; Polin & Moore, 1994). Finding that *S. crassipalpis* has a period of arhythmicity is not without precedent, as circadian oscillations cease in hibernating hamsters (Revel et al., 2007).

By the time of eclosion, rhythmic clock gene expression would be predicted to resume in response to cues entraining rhythmic eclosion. This study sought to discern what cues determine the eclosion rhythm during metamorphosis. Our findings indicate that thermoperiod determines eclosion rhythm, even when in contrast with photoperiod. Photoperiod alone did not provide synchronous eclosion by the Fisher exact test ($p<.05$). When using the parameter R (Winfree, 1970; Saunders, 1976; Smith, 1985) to measure the synchrony of eclosion, photoperiod was able to entrain synchronous eclosion, however the eclosion had a lower R value in groups with no thermoperiod or photoperiod than in groups with photoperiod alone, confirming that light is not an effective Zeitgeber during metamorphosis. This is in contrast to the two oscillator model described by Pittendrigh and Bruce (1959), suggesting that to determine eclosion rhythm *S. crassipalpis* follows a one oscillator model relying on thermoperiod.

The parameter used determine if synchrony is statistically significant determines at what life stage eclosion appears to be entrained. When using the parameter R, all pupal conditions resulted in synchronous eclosion, consistent with the results of Joplin & Moore (1999) who found photoperiod during the 3rd instar larvae entrains rhythmic eclosion. However, by the stricter statistical test of the Fisher's exact test, there is only synchronous eclosion in groups exposed to thermoperiod during metamorphosis. Given that parameter R values were the lowest

in groups that were exposed to thermoperiod during metamorphosis, there are clear effects of thermoperiod beyond the three day window at the end of the larval stage that was detected by Joplin & Moore (1999). It is also possible that the rhythmic eclosion pattern detected by Joplin & Moore (1999) was due to thermoperiod during metamorphosis rather than photoperiod during the third instar, because some flies can entrain off temperature oscillations as small as 0.4^{o}C, a temperature difference small enough to be feasibly induced by minor temperature changes in the rearing room (Zdarek & Denlinger, 1995). Finding that *S. crassipalpis* is consistent with the findings of Miyazaki et al. (2011).

The interaction of thermoperiod and photoperiod to determine eclosion rhythm is reflected in the expression of the clock gene *period*. When exposed to conflicting thermoperiod and photoperiod the expression of *per* mirrors the thermoperiod, even if in contrast to photoperiod. This is consistent with *D. melanogaster per* expression, which oscillates in a manner independent of the action of *tim*, and in response to thermoperiod (Yoshii et al., 2009). In *D. melanogaster cry* mutants still have rhythmic eclosion (Dolezelova, 2007). The action of CRY in conjunction with light explains the effect of photoperiod on the circadian clock, so if only thermoperiod determines eclosion rhythm, it is unsurprising that *cry* mutants retain rhythmic eclosion. Given the correlation of *per* expression with thermoperiod regardless of photoperiod and the entrainment of eclosion rhythm by thermoperiod regardless of photoperiod, *per* is a reasonable candidate for the clock gene upstream of the eclosion pathway. This is supported by the alteration of eclosion timing by changing *per* dosage and the elimination of *per* activity resulting in arrhythmic eclosion (Smith & Konopka, 1982; Smith & Konopka, 1981). The role of *per* in eclosion can be distinguished from that of *tim* by the independent function of PER and the rhythmic eclosion of *tim* mutants (Rothenfluh et al., 2000; Wuelbeck, 2005).

The function of the circadian clock in *S. crassipalpis* has ecological significance for the animal's survival. During larval stages behavioral periodicity could alter predator pressure, providing potential selective pressure for the maintenance of the circadian clock (Parecis-Silva, 2016). However from the time of pupariation until the time of eclosion the fly is immobile, and lacks overt behavior. The lack of circadian behavior is reflected in the lack of rhythmic clock gene expression. However, correct eclosion timing may be necessary for proper wing expansion and preventing desiccation (Pittendrigh, 1954, Tanaka & Watari, 2009). Synchronized eclosion may also play a role in changing predation patterns and facilitating mating (Gillott, 1995; Parecis-Silva, 2016). The clock gene *per* is a likely candidate for determining the time of eclosion, and the rhythmic expression of this clock gene has resumed by the time of eclosion in response to thermoperiod, the cue that determines eclosion rhythm. Overall, these findings suggest that the circadian clock of *S. crassipalpis* is variable throughout its life, correlating with changing behavioral needs.

VI CONCLUSION

The changes in circadian selective pressure, cues, and behavior throughout an animals life suggests that the circadian clock genes may have dynamic expression patterns based on the life stage of an organism. *Sarcophaga crassipalpis* were sampled at several life stages under varying thermoperiods and photoperiod using qRT-PCR and an eclosion assay to detect changes in clock gene expression pattern and the entrainment of circadian behavior. Our findings indicate that rhythmic clock gene expression ceases during the early pharate adult life stage, corresponding to a lack of overt behavior. Shortly before the rhythmic eclosion, rhythmic expression of the clock gene *period* has also resumed. Both eclosion rhythm and *period*

expression are entrained by thermoperiod, independent of photoperiod, suggesting that *period* may be the clock gene responsible for determining eclosion rhythm. Future studies into circadian rhythmicity should take into consideration the effect of life stage on clock genes expression and circadian behavior.

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